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Mode of Action of the Antibacterial Compound Dequalinium Acetate

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Dequalinium acetate is taken up rapidly by bacterial cells. Unlike the membrane-active drugs exemplified by cetrimide or chlorhexidine, its capacity for damaging the plamsa membrane is low. The drug appears to penetrate quite rapidly into the cytoplasm where its effect seems to be exerted. A review of the evidence obtained in this study suggests that nucleic acid-containing components of the cell may be the prime target of this compound.

Dequalinium acetate is an active antimicrobial agent, the properties of which were first described by Babbs et al. (2). The chemical, pharmacological, and antibacterial properties were described by Burgess (6), who concluded that the salts were stable and had a wide antimicrobial spectrum, including some penicillin-resistant staphylococci. The compound shows synergism with chlortetracycline, chloramphenicol, penicillin, streptomycin, and cetyltrimethyl-ammonium bromide. Among other members of the bisaminoquinaldinium series of compounds, some of the aspects of drug-cell interaction were studied by Cox (7, 8). The latter paper concluded that the drug caused little damage to the cytoplasmic membrane of the cell and that it was possible that the compound penetrated into the cytoplasm.

The present investigation provides further evidence for cytoplasmic penetration and also attempts to relate observed effects to the mechanism of the bacteriostatic and bactericidal actions of the compound.

MATERIALS AND METHODS

Organisms. *Escherichia coli* (type 1) NCTC 86, *Staphylococcus aureus* (Oxford strain), and *Bacillus megaterium* K.M. were used.

Chemicals. Dequalinium acetate (British Pharmacopoeia, p. 291, 1968), 1,1,decamethylenedi-(4-aminoquinaldinium acetate), hereafter referred to as dequalinium, is a white crystalline solid soluble in two parts of water at 25 C. The sample used was a gift from Allen and Hanbury Ltd., London, England. The chemicals used were of analytical reagent grade. Lysozyme and trypsin were from Koch-Light Laboratories, Colnbrook, Bedfordshire, England. Hexokinase, adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide phosphate (NADP) were from C. F. Boehringer and Soehne, Mannheim, Germany. Glucose-6-phosphate dehydrogenase was prepared by the method of Scott and Cohen (31).

Media. The nutrient broth was composed of 10 g of meat extract (Lemco; Oxoid), 10 g of peptone (Oxoid), 5 g of sodium chloride, and water to 1 liter. Solid media were prepared by the addition of 1.5% (w/v) agar (Oxoid no. 3). A drug-neutralizing (quenching) medium was prepared by adding 1% (w/v) Lubrol W (Imperial Chemical Industries Ltd., Blackley, England) and a 0.5% (w/v) vegetable lecithin (Wynmouth, Lehr and Fat Oils Ltd., London, England) to the nutrient broth. To this basic formula, lactose (10 g) and glucose (10 g) were added for use with *E. coli* and *S. aureus*, respectively. The final *p*H of all media was adjusted to 7.4.

Buffer solutions. Phosphate buffer, 0.067 M and *p*H 7.4, was prepared by mixing 19.6 ml of 0.067 M KH_2PO_4 with 80.4 ml of 0.067 M Na_2HPO_4. Citrate phosphate buffer was prepared as described by McIlvaine (22), and tris(hydroxymethyl)aminomethane (Tris) buffer was prepared by the method of Gomori (10).

Bacterial suspensions. A 5-ml amout of a 16-hr culture of the desired organism, grown in its appropriate nutrient broth at 37 C (*E. coli* and *S. aureus*) or at 30 C (*B. megaterium*), was inoculated onto 200 ml of the corresponding solid medium contained in a Roux flask and was incubated for 18 hr at the temperatures indicated above.

The cells were removed by washing with sterile phosphate buffer and were centrifuged at 1,000 × *g* to remove agar particles; the agar-free fluid was then centrifuged twice at 8,000 × *g* for 10 min. The dry weight content of the suspension was determined in a spectrophotometer (SP 500; Cambridge Instrument Co., England) at 420 nm by reference to a previously constructed calibration curve, dry weights for this curve being determined by drying suspensions in water to constant weight at 105 C. All bacterial weights hereafter refer to dry weights thus assessed.

Preparation of modified bacterial forms. Cells were treated in various ways by methods which might be expected to produce forms lacking a component or components of their cell walls.

Ethylenediaminetetraacetic acid (EDTA) treatment of E. coli. Suspensions (1 mg/ml) in 0.02 M Tris buffer, *p*H 8, were incubated at 20 C for 2 min with 10^{-3} M EDTA. Nine volumes of chilled nutrient broth was added to dilute the reactants, and the treated cells were recovered by centrifugation and suspended in 0.067 M phosphate buffer, *p*H 7.4. This treatment removes, at least in part, the lipopolysaccharide, lipoprotein component of the wall (19, 20). These forms were osmotically stable.

EDTA-lysozyme treatment of E. coli. A modification of the method of Repaske (30) was used. *E. coli* (10 mg/ml) was suspended in Tris buffer (0.03 M, *p*H 8) containing 0.5 M sucrose, 0.002% EDTA, and 20 μg of lysozyme per ml and was incubated at 30 C for 1 hr. The treated cells were deposited by gentle centrifugation and were resuspended in 0.067 M phosphate buffer (*p*H 7.4) containing 0.5 M sucrose. Microscopic examination of the final product revealed only spherical forms, approximately 1.5 μm in diameter.

Penicillin-induced spheroplasts of E. coli. Preparation was by the method of Lederberg (18). The spheroplasts were deposited by gentle centrifugation (12) after 2 to 3 hr at 37 C, when their number was maximal (13), and were resuspended in phosphate buffer (0.067 M, *p*H 7.4) containing 0.33 M sucrose.

Preparation of cell walls. Bacteria were disintegrated by shaking with glass beads in a Braun tissue disintegrator (25). Optimal conditions were determined experimentally and were as follows. *E. coli* (10 mg/ml) or *S. aureus* (6 mg/ml) suspended in 15 ml of Tris buffer (0.05 M, *p*H 7.4) was shaken with 50 g of glass beads, 0.1 mm in diameter, for 2 min at 2,800 strokes per min. The beads were removed by filtration through a sintered-glass bed of no. 1 porosity, and the walls were sedimented by centrifugation at 10,000 $\times$ *g* for 20 min. The crude walls thus obtained were incubated for 18 hr at 37 C in 5 ml of a solution of 0.5% (w/w) crystalline trypsin in 0.02 M Na_2HPO_4, *p*H 8.6. The trypsinized walls were washed three times in 0.85% NaCl and were finally suspended in 0.067 M phosphate buffer, *p*H 7.4.

Determination of minimal inhibitory concentration (MIC). Graded doses of dequalinium were prepared in 10 ml of nutrient broth and were inoculated with suspensions of *E. coli* or *S. aureus*. The presence or absence of growth was noted after 24 hr of incubation at 37 C. Inoculum levels of from 10^2 to ca. 10^8 cells/ml were used for each determination of the MIC. Because of the intrinsic turbidity caused by higher inoculum levels (10^8 and above), evidence of growth or bacteristasis was obtained by performing total counts with a Thoma counting chamber, 0.02 mm deep.

Determination of bactericidal activity. As dequalinium causes clumping of organisms, the bactericidal effect was determined as a mean single survivor time (MSST) calculated by the method of Mather (24), from extinction data derived from the method of Berry and Bean (4), employing 20 replicates and 5 ml of quenching medium, which was previously shown to neutralize residual dequalinium satisfactorily.

Analytical methods. We used the following analytical methods.

(i) Dequalinium was determined directly by measuring absorbancy at 327 nm or by the colorimetric method of Ballard, Isaacs, and Scott (3).

(ii) To determine cellular exudates, material absorbing at 260 nm was estimated by direct spectrophotometry, after first removing dequalinium (which also absorbs at 260 nm), when present, by passing through a column (6 by 1 cm) of a polyacrylamide gel (bio-gel P-2 100/200 mesh, Bio-Rad Laboratories, Richmond, Calif.), thereby obtaining complete quantitative separation. Pentoses were determined by the method of Mejbaum (26), providing that the dequalinium content of the system did not exceed 80 μg/ml; above this concentration interference occurred.

(iii) Turbidity changes in bacterial suspensions were determined by absorbancy at 420 nm.

(iv) For glucose respiration studies, oxygen uptakes were determined by the direct method of Warburg in an atmosphere of air at 100 oscillations/min. Each flask contained, in 3 ml, 3 mg of cells, 0.0067 M phosphate buffer (*p*H 7.4), and 0.02 M glucose. Dehydrogenase activity was determined by (i) measuring the reduction of triphenyltetrazolium bromide (TTB) in open tubes (11) in a system containing 5 mg of cells, 250 μg of TTB, and 0.0067 M phosphate buffer (*p*H 7.4) in 5 ml. At the completion of an experiment, an equal volume of acetone was added followed by the deposition of cells by centrifugation. The triphenylformazan produced by reduction of TTB was determined colorimetrically at 525 nm. (ii) Dehydrogenase activity was also determined by measuring the reduction of methylene blue in evacuated Thunberg tubes. For whole cells, the reaction system contained, in 5 ml, 0.02 M glucose, 0.0067 M phosphate buffer (*p*H 7.4), 10^{-4} M methylene blue, and 0.375 mg of cells. For the cell-free oxidation of glucose, the system contained, in addition, 2×10^{-7} M hexokinase, 0.00032 M ATP, 0.00026 M NADP, and a sufficient volume of the cell-free preparation to give a reduction rate equal to that of the whole-cell system. To all of these systems the appropriate quantity of drug was added, as required, without altering the final volumes quoted.

(v) To analyze dequalinium-precipitated bacterial cytoplasmic constituents, nucleic acid was estimated as the material absorbing at 260 nm. Protein was determined according to the method of Kabat and Mayer (16).

Determination of electrophoretic mobilities. A rectangular cell, 5 by 1.3 cm and with a depth of 600 μm, was connected to a copper-copper sulfate electrode system. Apart from details of cell dimensions and the electrode system, the apparatus resembled that described by Lerche (21). Stationary levels in the cell were calculated by the method of Komagata (17), and the potential gradient in situ in the cell was determined as described by McQuillen (23) from a knowledge of the specific resistance of the bacterial suspension being used. Mobilities were determined in phosphate buffer (*p*H 7.4; ionic strength, 0.01) at 20 C with cell densities in all cases of 0.1 mg/ml.

Determination of dequalinium uptake. Cells or cell walls were left in contact with solutions of dequalinium under varying conditions of time, *p*H, and ionic environment, and then were quickly deposited by centrifugation at 5,000 $\times$ *g* for 5 min. Clarification of the supernatant fluid was accomplished by a further centrifugation (10,000 $\times$ *g* for 10 min). The residual

dequalinium was determined by the colorimetric procedure.

Preparation of wall-free B. megaterium cells. *B. megaterium* cells (1.0 mg/ml) in 0.0067 M phosphate buffer, after treatment with varying concentrations of dequalinium at 20 C for 30 min, were deposited by centrifugation, washed, and suspended in the same buffer. A 1-ml amount of this suspension was added to 9 ml of a solution to give the following final concentrations: lysozyme, 20 μg/ml; sucrose, 0.5 M; and cells, 0.2 mg/ml. After 3 hr of contact, samples were removed and examined microscopically.

TABLE 1. *Mean single survivor times of E. coli and S. aureus in the presence of varying concentrations of dequalinium*

Dequalinium	MSST (min)		Ratio of *S. aureus* to *E. coli*
	E. coli	*S. aureus*	
μg/ml			
800	26	90.7	3.49
600	55.25	184	3.33
500	95.5	300	3.14
300	191	536	2.81

RESULTS

The MIC values at the various inoculum levels, expressed as micrograms per milliliter, were 4.5 (10^2 cells/ml), 6.5 (10^4 cells/ml), 8.0 (10^6 cells/ml), and 13.0 (1.2×10^8) for *E. coli* and 0.26 (10^2 cells/ml), 0.38 (10^4 cells/ml), 0.60 (10^6 cells/ml), 1.80 (1.2×10^8 cells/ml) for *S. aureus*.

By subculturing a loopful of medium from tubes showing no visible growth into an additional 10 ml of medium, an approximate bactericidal end point was obtained. For inocula having up to 10^6 cells/ml, these values were within 0.5 μg/ml of the MIC value. At the higher inoculum level of 1.2×10^8 cells/ml, more varied values were obtained, but were within 1 to 3 μg/ml of the MIC.

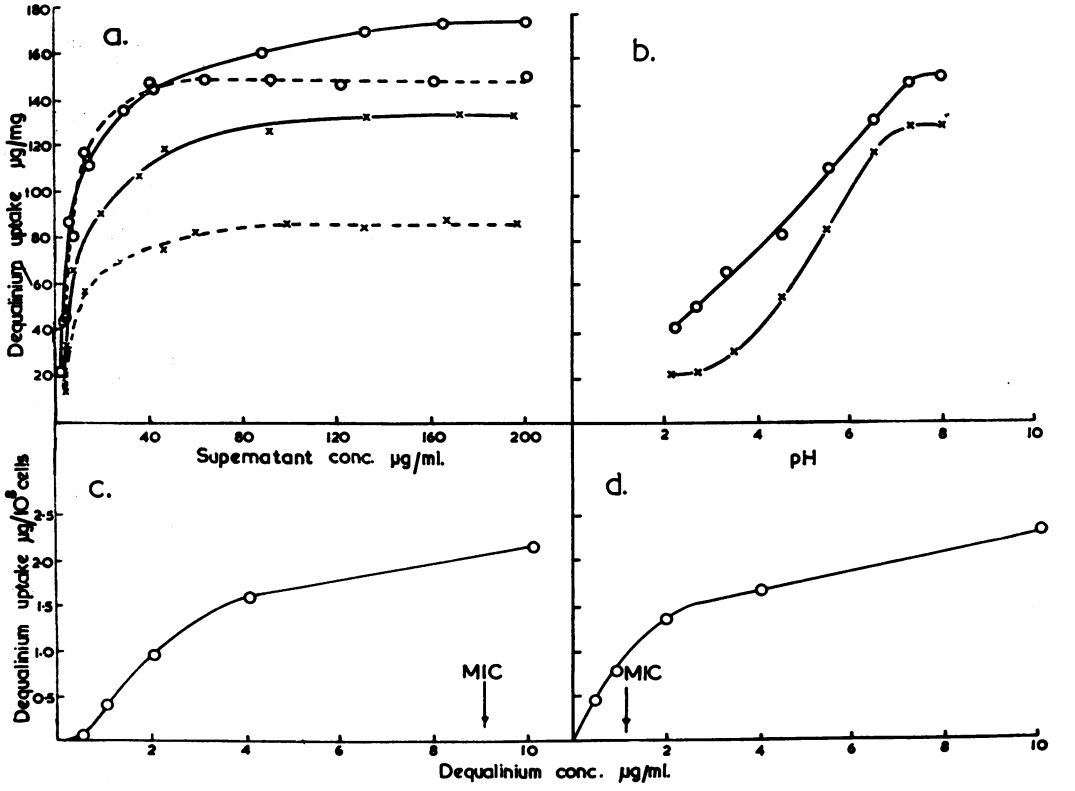

FIG. 1. *(a) Adsorption isotherms for uptake of dequalinium by E. coli (×) and S. aureus (○) suspensions (1 mg per ml) after 10 min of contact at 20 C from distilled water (dashed line) or 0.0067 M phosphate buffer, pH 7.4 (solid line). (b) Effect of pH on adsorption of dequalinium by E. coli (×) and S. aureus (○) suspensions (1 mg per ml) from 0.0067 M McIlvaine's citric acid-phosphate buffer after 10 min of contact at 20 C. (c and d) Uptake of dequalinium by 10^8 E. coli and 10^8 S. aureus cells per ml, respectively, from 0.0067 M phosphate buffer, pH 7.4, containing 0.5% sodium chloride after 30 min of contact at 20 C. MIC determined for 10^8 cells per ml in nutrient broth after 24 hr of incubation at 37 C.*

Bactericidal activity. MSST values at various concentrations are shown in Table 1. There was a linear relationship between the log of MSST and the log of dequalinium concentration over the above concentration ranges.

Uptake of dequalinium by bacterial cells. Isotherms for the uptake of dequalinium from distilled water and phosphate buffer by whole cells of *E. coli* and *S. aureus* are Langmuirian (Fig. 1a). The effect of *p*H on uptake over the *p*H range of 2 to 8.5 was determined by repeating the experiments in McIlvaine's buffer after ascertaining that the colorimetric determination was valid in this buffer (Fig. 1b).

Uptake was also affected by buffer strength. Uptakes from 0.0067 and 0.067 M phosphate buffer, *p*H 7.4, were 129 and 115 μg/mg for *E. coli* and 159 and 120 μg/mg for *S. aureus*.

Newton (29), following an experiment of Bundenberg de Jong (5), has shown that some indication of the chemical nature of drug binding sites may be obtained by investigating competition for uptake of that drug by a series of ions. Dequalinium uptake was measured in 0.005 M Tris buffer, *p*H 7.4, containing Ca^{2+}, Mg^{2+}, Ba^{2+}, or Sr^{2+} (10 μmoles/ml) or UO_2^{2+}, Ce^{3+}, or La^{3+} (0.2 μmole/ml). The competition pattern shown by Ce^{3+}, La^{3+}, and UO_2^{2+} indicated that carboxyl groups might be involved in dequalinium uptake. Results with the remaining (divalent) cations indicated that a phosphate binding site might also be involved.

Uptakes of dequalinium by the purified cell wall preparations, adjusted to contain the amount of wall material expected in a whole-cell suspension containing 1 mg/ml, were determined in 0.0067 M phosphate buffer, *p*H 7.4, at 20 C for 10 min. From initial dequalinium concentrations of 10, 50, 100, and 200 μg/ml, *S. aureus* walls took up 7.0, 31.5, 31.8, and 32.5 μg, representing

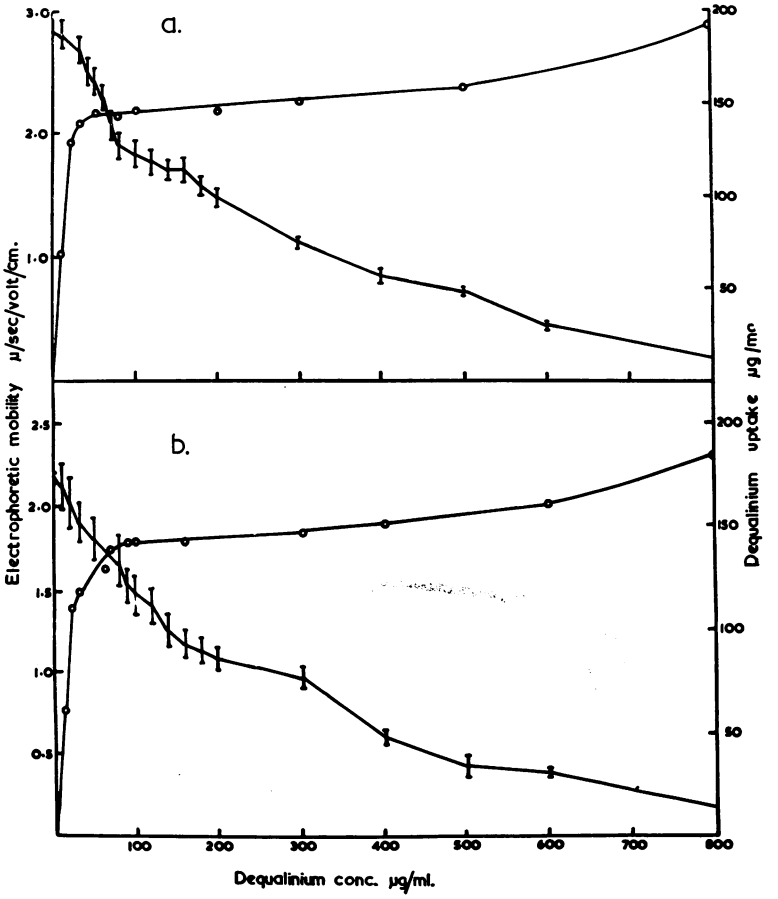

FIG. 2. *Effect of dequalinium on the electrophoretic mobility of bacterial cells (0.1 mg per ml) in phosphate buffer (μ = 0.01; pH 7.4) after 10 min of contact at 20 C. (a) E. coli. (b) S. aureus. Symbols: bars, electrophoretic mobility; O, adsorption of dequalinium determined under identical conditions.*

72, 65, 33, and 21% of the whole cell uptake, and *E. coli* walls took up 1.64, 13.0, and 15.0, and 16.1 μg, representing 27, 27, 18.5, and 11.5% of the whole cell uptake.

To compare drug uptake and MIC values, the latter were redetermined in broth with an inoculum of 10^8 cells/ml; the new MIC values (μg/ml) were 9.0 for *E. coli* and 1.2 for *S. aureus*. At these values, equilibrium uptakes (μg/mg) from phosphate buffer, *p*H 7.4, plus 0.5% NaCl (dequalinium could not be determined in nutrient broth) were 80 for *E. coli* and 54 for *S. aureus*.

Effect of dequalinium on electrophoretic mobility. Dequalinium caused a steady reduction in the net negative charge of both *E. coli* and *S. aureus* with increasing concentration, but not to the point of reversal (Fig. 2a, b). Washing the drug-treated suspension removed very little of the drug. For example, a suspension which had taken up 128 μg/mg lost, on four successive washings, 2.5, 5.2, 8.6, and 5.0 μg/mg; i.e., a total of 21.3 μg/mg or 16.6% of the original. However, these washings had the effect of restoring the mobility of the treated suspension to that of untreated cells.

Mobilities determined as quickly as possible, i.e., within 4 min of treatment, were the same as after 30 min of contact.

Dequalinium-induced changes in suspension turbidity. The drug caused a steady concentration-dependent increase in the turbidity of whole-cell suspensions; this increase was less rapid in *E. coli* than in *S. aureus* (Fig. 3a, b; results for 10 min and 3 hr of contact).

The turbidity of EDTA-lysozyme spheroplasts of *E. coli* changed within 10 min and remained constant for periods in excess of 3 hr. The drug also caused a concentration-dependent increase in the turbidity of isolated walls, but there was no evidence of wall dissolution.

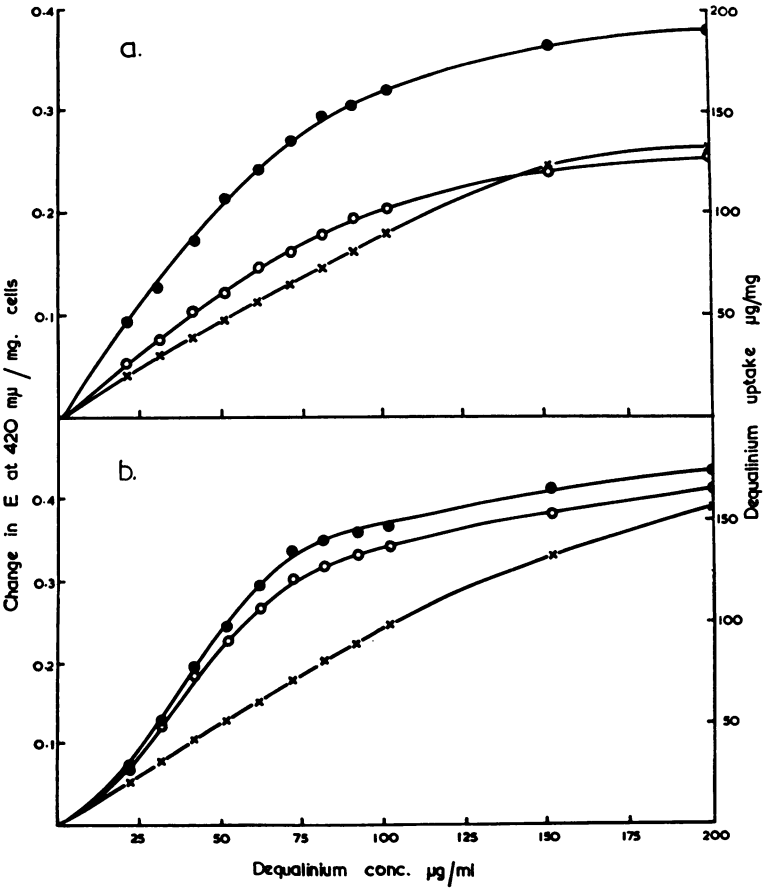

FIG. 3. *Effect of dequalinium on optical density of bacterial suspensions (1 mg per ml). (a) E. coli. (b) S. aureus. Extinction determined at 420 nm against a reference cell containing 1 mg of cells per ml after 10 min* (○) *and 180 min* (●) *of contact at 20 C in 0.0067 M phosphate buffer, pH 7.4. Dequalinium uptake after 10 min of contact* (×).

Metabolic studies. The effect of dequalinium on aerobic and anaerobic metabolism of glucose is shown in Fig. 4a and b. There is little difference in the concentration-inactivation curve for these two processes, and stimulation of neither was observed.

A comparison was made of the effect of methylene blue reduction in whole cells and in a reconstructed cell-free preparation; again, glucose was the substrate. The whole-cell system was found to be much more sensitive (Fig. 4c, d).

Loss of cytoplasmic constituents. Dequalinium-treated *E. coli* and *S. aureus* lost pentoses and material absorbing at 260 nm (Fig. 5), the amount

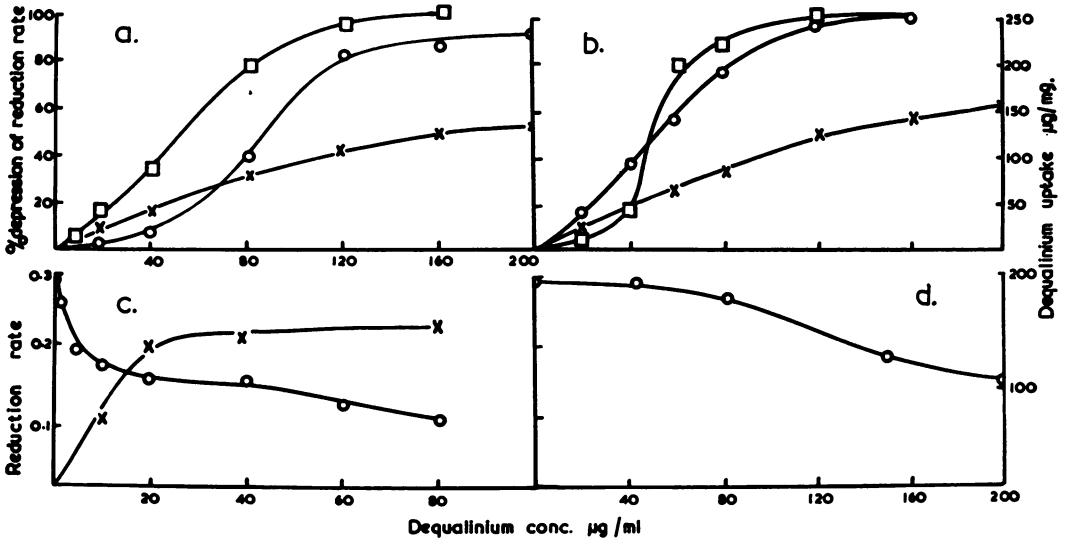

FIG. 4. *(a and b) Effect of dequalinium on the rate of oxygen uptake* (○) *and the rate of TTB reduction* (□) *by E. coli and S. aureus, respectively. (c and d) Effect of dequalinium on the rate of reduction of methylene blue* (○) *by E. coli and by partially purified glucose-6-phosphate dehydrogenase from E. coli, respectively. Dequalinium uptake* (×).

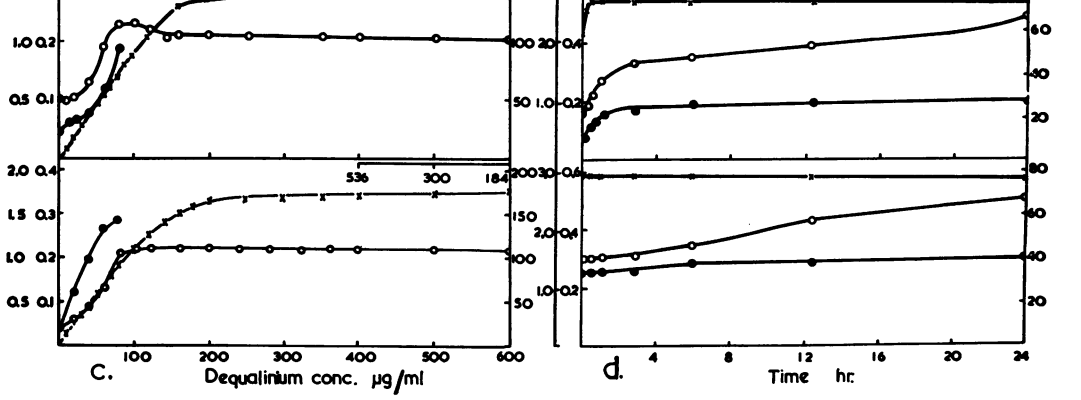

FIG. 5. *(a and c) Effect of dequalinium concentration on leakage of material absorbing at 260 nm and on leakage of pentose from E. coli and S. aureus suspensions (1 mg per ml) after 180 min of contact in 0.0067 M phosphate buffer (pH 7.4) at 20 C. (b and d) Effect of time on leakage of material absorbing at 260 nm and on leakage of pentose from E. coli and S. aureus suspensions (1 mg per ml) at 20 C in 0.0067 M phosphate buffer (pH 7.4) containing 80 µg of dequalinium per ml. Symbols:* ○, *E at 260 nm;* ●, *pentose;* ×, *uptake of dequalinium. (A) Pentoses, µg/mg. (B) E, 260 nm/mg. (C) Uptake of dequalinium, µg/mg. Also shown are MSST values.*

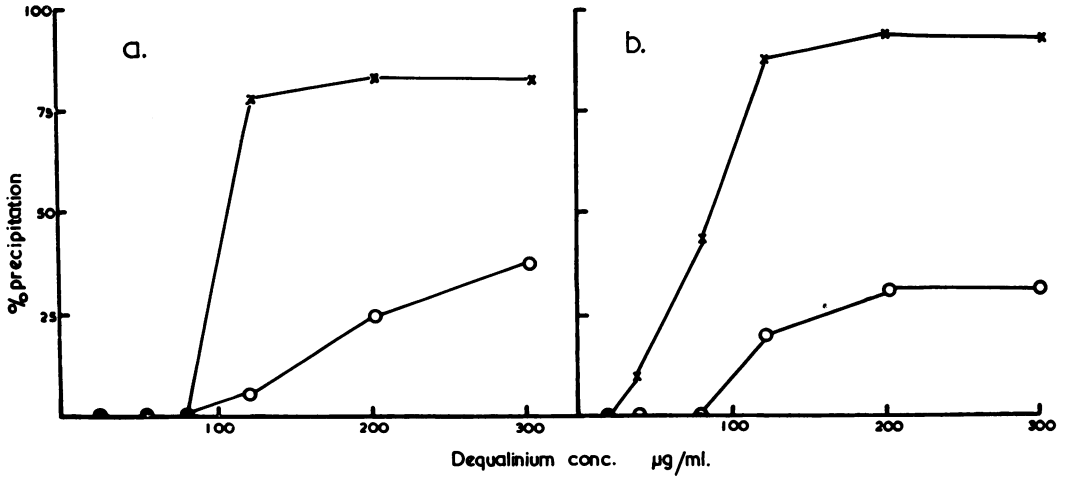

FIG. 6. *Effect of dequalinium on precipitation of nucleic acid* (×) *and protein* (○) *derived from 1 mg of (a) E. coli or (b) S. aureus per ml after 180 min of contact at 20 C.*

TABLE 2. *Effect of dequalinium pretreatment on the conversion of B. megaterium to protoplasts by lysozyme*

Dequalinium concn	Microscopic appearance after 3 hr of contact with lysozyme
µg/ml	
0	Spherical forms (protoplasts)
5	Spherical forms
10	> 95% Spherical forms
20	> 95% Spherical forms
50	> 95% Spherical forms
100	Rods beginning to appear
200	Ca. 50% remaining as rods
300	Rod forms only

of both materials reaching a miximum at a drug concentration of 80 µg/ml. With this concentration, the effect of time on the course and nature of leakage at 4, 20, and 37 C was investigated. Leakage, as with adsorption, took place rapidly with *S. aureus*, but both processes were slower with *E. coli*. In general, the amount of material leaking increased with increasing temperature. Leakage from both organisms increased slowly with time. Leakage from EDTA-treated *E. coli* took place more rapidly than from normal cells

Precipitation of cytoplasmic constituents. Protein and nucleic acid precipitated from the constituents of both organisms are shown in Fig. 6a and b; it can be seen that nucleic acids are more sensitive to precipitation than protein.

Some material extracted from cells maintained at 98 to 100 C for 10 min was precipitated by dequalinium concentrations greater than 100 µg/ml. Simple aqueous solutions of nucleotide triphosphates were precipitated at a similar order of concentration. Dequalinium had no precipitating effect on aqueous solutions of purines, pyrimidines, or nucleotide monophosphates.

The effect of pretreating the rod-shaped *B. megaterium* with dequalinium and then attempting to convert the rods to spherical protoplasts with lysozyme in sucrose-stabilized buffer is shown in Table 2.

DISCUSSION

When cell suspensions are placed in contact with dequalinium solutions, the drug is taken up by the cells, which may be killed or prevented from dividing. The adsorption of dequalinium is Langmuirian. Giles et al. (9) argued that this type of isotherm indicates that adsorbate molecules are first accepted readily by the adsorbant, but the chance for further adsorption decreases as available sites become filled; i.e., there is no tendency for multilayer formation.

Certain experiments yield information regarding the site of adsorption of dequalinium. The fact that Langmuir's adsorption equation may be applied to the experimental situation found would normally indicate monomolecular layer adsorption. However, consideration of molecular area and available bacterial surface area indicates that considerably more drug is adsorbed at saturation level than can be accommodated in a monomolecular layer. Cox (8) studied 1-decamethylene-4-aminoquinaldinium and concluded that sufficient drug was adsorbed at the saturation level to correspond to at least 30 monolayers around the bacterial cell. It must be concluded that adsorption takes place at sites separate from

the cell surface, either within the cell wall or at the cell membrane, or that penetration into the cytoplasm occurs.

The location of the dequalinium binding site is indicated by the effects of *p*H, buffer strength, and competition with certain cations on dequalinium adsorption. Albert (1) discussed the effect of *p*H on heterocyclic quaternary ammonium compounds, indicating how they can combine covalently with hydroxyl ions in alkaline conditions to produce pseudobases. Such compounds are highly lipid-soluble, and possibly the increase in *p*H increased the lipid solubility of dequalinium. However, *E. coli* adsorbs less dequalinium than *S. aureus* but contains more lipid. Therefore, it seems likely that the variation in drug uptake with *p*H is due to a variation in the degree of ionization of the cell surface. An electrostatic binding mechanism is also suggested because drug uptake is lower in buffers of high molarity, possibly due to competition for available sites. From a study of the depression of dequalinium adsorption by a cationic series, it seems possible that both ionized phosphate and carboxyl groups are involved in drug uptake.

Measurements of dequalinium uptake by isolated cell wall preparations could indicate that, in the whole cell, drug uptake occurs at sites separate from the cell wall. Alternatively, isolation of the cell walls may destroy some of the sites available for reaction with dequalinium. The saturation adsorption level for whole cells and modified forms of *E. coli* was the same in all cases, although the rate at which equilibrium was attained differed. This further indicates that the cell wall is not the prime site of adsorption, although it may constitute a permeability barrier to drug uptake.

Further evidence for the site of dequalinium adsorption being separate from the cell surface was obtained from a study of the electrophoretic mobility of drug-treated cells. The overall negative charge on the bacterial cell was steadily reduced, but even at the highest drug concentrations used it was never abolished or reversed. Reversal of charge would certainly be expected with highly localized concentrations of a positively charged compound, particularly if this concentration was a buildup of multilayers of drug around the cell. In the case of *E. coli*, the rate of change of electrophoretic mobility was more rapid than could be detected by the techniques used, being more rapid than adsorption, suggesting that equilibrium takes place rapidly at the cell surface, whereas adsorption is still proceeding at other sites. As washing drug-treated cells restores electrophoretic mobility to that of untreated controls, even though the amount of drug removed is very small, it seems likely that only a small proportion of drug is held at the cell surface, which is the part of the cell accessible to electrophoretic study.

Several mechanisms may be involved in the turbidity changes resulting from dequalinium uptake. Adsorbed drug may itself cause a change in the reflecting surface of the bacterial cell, or it may alter the cell surface so that its reflecting properties are changed. The drug may cause a change in cell size or, alternatively, may penetrate to the interior of the cell and change the refractive index of its contents. The difference in rate of change observed between *E. coli* and *S. aureus* and the rapid change observed in EDTA-lysozyme spheroplast suspensions of *E. coli* suggest that the cell wall of the gram-negative organism constitutes a permeability barrier to dequalinium.

Experiments show that little difference exists between the drug concentrations required to inhibit aerobic metabolism of glucose and those required to inhibit anaerobic metabolism of glucose, indicating that the cytochrome system is not specifically attacked. With tetrachlorosalicylanilide, Woodroffe and Wilkinson (32) found a 20-fold difference between the concentrations of the compound required to inhibit glucose fermentation and the concentrations required to inhibit glucose oxidation. They concluded that the compound specifically attacked enzymes involved in aerobic metabolism. The dehydrogenase activity of whole cells was more sensitive to the action of dequalinium than was that of an isolated enzyme system. Because of the concentration differences, it seems likely that different mechanisms are involved. The enzyme system is likely to be inhibited by direct denaturation of one of its components (e.g., ATP or protein). Figures 4 and 6 indicate that protein denaturation and loss of enzyme activity are similar. Loss of activity in a whole-cell system may be due to a change in permeability or to a loss of spatial integration of the various systems involved (27). No evidence was found for a stimulation of dehydrogenase activity in the presence of low dequalinium concentrations. With the compound chlorhexidine, Hugo and Longworth (14) found a marked stimulation of the rate of dehydrogenase activity in the presence of low concentrations of the compound. These concentrations corresponded to those causing a high level of leakage from bacterial cells, and it was concluded that an increase in cell permeability was responsible for the increased dehydrogenase activity. Leakage levels were low at all dequalinium concentrations studied. There is the suggestion of a peak in the curve obtained for *E. coli*, but this is small when compared with the results of Newton (28) using

polymyxin or Hugo and Longworth (13) using chlorhexidine. Low leakage levels could indicate that the compound has a low membrane-disrupting capacity, suggested by the absence of lysis of protoplasts or spheroplasts. Alternatively, dequalinium may penetrate into the cytoplasm and cause precipitation of certain compounds, thus preventing their leakage into the medium. However, some compounds which are likely to leak after drug treatment (nucleotides; see reference 28) are not precipitated from solution by dequalinium. Dequalinium could seal the membrane at low drug concentrations, thus preventing leakage, but protein precipitation is the likely mechanism for this, and dequalinium is not a highly active protein precipitant. Low leakage levels could not be attributed to an inhibition of an autolytic process because, at a concentration of 80 μg/ml, leakage was higher at 37 C than at 2 C; also, leakage continued to increase with time, indicating that autolytic activity was proceeding in the presence of the drug.

Nucleic acids are considerably more susceptible to precipitation by dequalinium than are proteins. The lack of effect of serum on the MIC of dequalinium against *S. aureus*, reported by Babbs et al. (2), indicated that the compound was not strongly bound to protein. That precipitation of the cytoplasm occurs in the whole cell is indicated by the fact that *B. megaterium* cells treated with a high concentration of dequalinium cannot undergo the transformation to spherical protoplasts on treatment with lysozyme.

It is possible to make some generalizations regarding the mechanism of inhibitory and bactericidal actions of dequalinium. There is a substantial difference between the MIC values determined for *E. coli* and *S. aureus*, following the general pattern that such compounds are more effective against gram-positive than against gram-negative organisms. However, when uptakes were determined under inhibitory conditions, the levels of uptake (μg/mg) were not dissimilar, indicating a difference in affinity for the compound rather than a different mechanism of action. Inhibitory levels occur in the concentration range wherein leakage is still concentration-dependent and precipitation of cytoplasmic constituents is low. Metabolic activity is considerably reduced.

At higher concentrations, i.e., concentrations which are more rapidly bactericidal, leakage is no longer concentration-dependent, but precipitation of cytoplasmic constituents reaches a maximum. It is possible that the compound owes its bactericidal action to the penetration into the cytoplasm followed by precipitation of cytoplasmic material, nucleic acid seeming the most sensitive.

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